Andreia Filipa Ramos Paciência

Licenciada em Farmácia

Optimization of techniques for rapid assessment of inflammatory responses

Dissertação para obtenção do Grau de Mestre em Bioquímica para a Saúde

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UNIVERSIDADE NOVA DE LISBOA

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Optimization of techniques for rapid assessment of inflammatory responses

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Abstract

Inflammation is a defence mechanism involving the immune system, in response to infection or other types of cell damage. In acute inflammation, immune cells migrate to the injury site, become activated and express pro-inflammatory cytokines to rapidly eliminate the pathogenic factors. Chronic inflammation may be preceded by the persistence of acute inflammation and involves mononuclear invasion that causes tissue damage or repair. Chronic inflammation is now thought to be a process underlying a significant percentage of disease-related deaths worldwide. Therefore, it is important to develop methods to identify inflammation of patients in the least invasive way.

The current work aims to optimise a protocol to investigate inflammation biomarkers in patients suffering from chronic diseases. We hypothesised that these biomarkers could be addressed by analysing patients blood samples or fibroblast stimulated with mitogen or pro-inflammatory cytokines.

To optimise the stimulation protocol, we tested different concentrations of PMA with Ionomycin, TNFα and lipopolysaccharide (LPS) to obtain effective cellular stimulation in the shortest time possible, preserving cell viability. For the blood sample, we evaluated cell surface biomarkers, CD69 and CD154 and the expression of the pro-inflammatory cytokine IFNγ by flow cytometry while for fibroblasts we analysed the expression of IL-6 and FGF-2 by RT-qPCR.

Our results showed that the ideal concentration of stimulus used for evaluation of whole blood samples is 50 ng/ml of PMA + 1µg/ml of ionomycin for cytokine evaluation and 5,5 ng/ml of PMA + 0,05 µg/ml of ionomycin for cell surface markers evaluation. Moreover, the optimal concentration for dermal fibroblasts stimulation is 100 ng/ml of TNFα, 25 ng/ml of PMA + 3 µg/ml ionomycin and 1 µg/ml of LPS. We were able to observe the expression of all biomarkers tested and their modulation upon stimulation.

This work helps to demonstrate that a rapid protocol can be used to evaluate inflammatory biomarkers in blood samples and that fibroblast can be a reliable alternative to evaluate inflammatory stimulation. These protocols are essential to screen for inflammation biomarkers in diseases such as osteoarthritis, cancer, asthma, among others.

Keywords: Flow cytometry, Immune cells, Chronic inflammation, Fibroblasts; Biomarkers

Resumo

A inflamação é um mecanismo de defesa que envolve o sistema imunitário, em resposta à infeção ou a outros tipos de danos celulares. Na inflamação aguda, as células imunes migram para o local da lesão, tornam-se ativadas, expressam citocinas pró-inflamatórias para eliminar rapidamente os fatores patogénicos. A inflamação crónica pode ser precedida pela persistência da inflamação aguda e envolve a invasão mononuclear que causa dano ou reparo do tecido. A inflamação crónica é atualmente considerada um processo subjacente a uma percentagem significativa de mortes relacionadas a doenças em todo o mundo. Por conseguinte, é importante desenvolver métodos para identificar a inflamação nos pacientes da forma menos invasiva.

O presente trabalho tem como objetivo otimizar um protocolo para investigar biomarcadores de inflamação em pacientes portadores de doenças crónicas. A nossa hipótese recaiu sobre se os biomarcadores utilizados poderiam ser direcionados para a análise de amostras de sangue de doentes ou fibroblastos estimulados com mitogénio ou citocínas pró-inflamatórias.

Para otimizar o protocolo de estimulação, testamos diferentes concentrações de PMA com Ionomicina, TNFα e lipopolisacárido (LPS) para obter uma estimulação celular eficaz no menor tempo possível, preservando a viabilidade celular. Para a amostra de sangue, avaliamos biomarcadores de superfície celular, CD69 e CD154 e a expressão da citocina pró-inflamatória IFNγ por citometria de fluxo, enquanto para fibroblastos analisámos a expressão de IL-6 e FGF-2 por RT-qPCR.

Os resultados mostraram que a concentração ideal de estímulo utilizada para avaliação de amostras de sangue total é de 50 ng/ml de PMA + 1 µg/ml de ionomicina para avaliação de citocinas e 5,5 ng/ml de PMA + 0,05 µg/ml de ionomicina para avaliação de marcadores celulares de superfície. Além disso, a concentração ideal para estimulação de fibroblastos dérmicos é de 100 ng/ml de TNFα, 25 ng/ml de PMA + 3 µg/ml de ionomicina e 1 µg/ml de LPS. Conseguimos observar a expressão de todos os biomarcadores testados e a sua modulação após estimulação.

Este trabalho ajuda a demonstrar que um protocolo rápido pode ser usado para avaliar biomarcadores inflamatórios em amostras de sangue e que os fibroblastos podem ser uma alternativa confiável para avaliar a estimulação inflamatória. Estes protocolos são essenciais para rastrear biomarcadores de inflamação em doenças, como osteoartrite, cancro, asma, entre outras.

Palavras-chave: Citometria de fluxo, Células imunes, Inflamação crónica, Fibroblastos, Biomarcadores

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List of Abbreviations

CDG - Congenital Disorders of Glycosylation

$CD4^+$ T Cells - Helper T Lymphocytes Cells

$CD8^+$ T Cells - Cytotoxic T Lymphocytes Cells

DAG - Diacylglycerol

DAMPs – Damage-Associated Molecular Patterns

DMSO – Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

ECM - Extracellular Matrix Components

FBS – Fetal Bovine Serum

FGF2 - Fibroblast Growth Factor-2

FITC – Fluorescein isothiocyanate

FSC – Forward Scatter

IFNγ – Interferon Gamma

IL6 – Interleukin-6

IP_3 - Inositol Triphosphate

LPS – Lipopolysaccharide

MFI – Median Fluorescence Intensity

MHC – Major Histocompatibility Complex

MMPs - Induce Matrix Metalloproteinases

NK Cells – Natural Killer Cells

PAMPs - Pathogen-Associated Molecular Patterns

PBS – Phosphate Buffered Saline

PI(4,5)P2 - Phosphatidylinositol 4,5 Bisphosphate

PKC - Protein Kinase C

PMA - Phorbol 12-myristate-13-acetate

PRRs - Pattern Recognition Receptors

RBCL - Red Blood Cells Lysis Buffer

RNA - Ribonucleic Acid

RT-qPCR - Real-Time Quantitative Polymerase Chain Reaction

SSC – Side Scatter

TCR - T cell receptor

TIMPs - Tissue Inhibitor Of Metalloproteinases

TNFα – Tumor Necrosis Factor Alpha

1. Introduction

1.1 Immune system

The immune system is essential for human body survival. This system has an important role in homeostasis, and comprises a wide variety of cells, organs, tissue, and molecules, is capable of protection against foreign attack, potentially from the pathogenic microorganism, while also removing diseased cells and abnormal endogenous materials.[1–3]

When this balance is disrupted, it can lead to a weakened function and lead to infections. However, an unbalanced and excessive immune response leads to pathologies, such as allergies and autoimmune diseases.[1,4]

1.1.1 Immune system cells

The immune system consists of two different parts, the innate and adaptive immunity. Innate immunity is mediated through basophils, eosinophils, neutrophils, mast cells, natural killer cells (NK cells), macrophages, and dendritic cells. The macrophages and dendritic cells are responsible for presenting antigens to the cells of the adaptive immune system, and for that reason are the only cells that mediate both innate and adaptive immune systems. The adaptive immune system is mediated through two types of cells, known as lymphocytes. B lymphocytes are responsible for the humoral immunity while T lymphocytes drive the cellular immunity, and both cells are mainly dependent on specific antigens.[1,5]

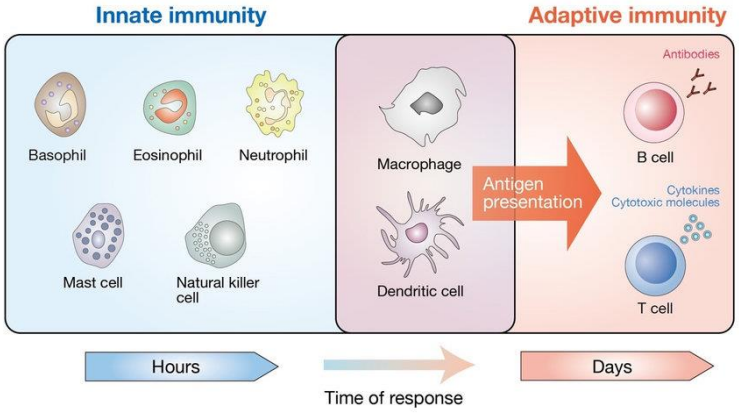


Figure 1.1 - Cellular components of the mammalian immune system: *The immune system consists of two distinct parts, innate and adaptive immunity. Basophils, eosinophils, neutrophils, mast cells, natural killer cells, macrophages, and dendritic cells mediate the innate immunity. The adaptive immune system refers to an antigen-specific defence mechanism that takes several days to develop but provides long-lasting protection. The adaptive immune system includes B cell-mediated humoral immunity and T cell-mediated cellular immunity, both of which are directed towards the specific antigens. Macrophages and dendritic cells are unique subsets that have both innate and adaptive immune cell traits. As professional antigen-presenting cells, macrophages and dendritic cells are critical in the induction of adaptive immunity by presenting the antigens to antigen-specific T and B lymphocytes.*[5]

1.1.2 Innate immune system

The immune response is composed of three complementary parts where the innate immune system is the second line of defence. This response is almost immediately activated through chemical signals sent from the epithelial surface barrier, the first line of defence.[1,6]

According to Newton[7], there are innate immune cells, such as macrophages, fibroblasts, mast cells, and dendritic cells, that can reside in the tissues or that circulate through the bloodstream, such as leukocytes, including monocytes and neutrophils. They recognize pathogen invasion or cell damage with intracellular or surface-expressed pattern recognition receptors (PRRs). These receptors detect, either directly or indirectly, pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins, and carbohydrates, or damage-associated molecular patterns (DAMPs).[3,6–8] After recognition, the expression of chemokines, cytokines and other factors, promotes activation and recruitment of immune cells. Once activated, these cells are responsible for the destruction of the pathogen.[1,6,9]

When the innate immune system is no longer able to deal with an infection, and when pathogens entered cells, the PRRs can detect and send signals through, for example, cytokines or chemokines and the adaptive immune system is activated by major histocompatibility complex (MHC) by antigen-presenting cells.[1,6]

1.1.3 Adaptive immune system

The adaptive immune system is a secondary response, with a longer time in the intervention compared to the innate immune system and can take hours or even days.[1,6] The immune response of the adaptive system is a highly strong and specialized response, and besides pathogen recognition is also involved in other inflammatory processes such as allergies, autoimmunity diseases, and even in rejection of tissue grafts.[9] This system is composed by two major populations of lymphocytes which can recognize a complex number of antigens (Figure 1.2.).[1]

The B lymphocytes are responsible for the humoral immunity, the principal defence mechanism. These cells produce molecules called antibodies with the capacity to recognise pathogens outside of cells.[1] They also contribute as antigen-presenting cells for T lymphocytes activation, being able to express antigens via MHC Class II and which are binding antigenic peptides detected by T Lymphocytes and recognize co-stimulatory molecules.[10,11] The B lymphocytes are implied in two types of response: the primary immune response characterized by a first exposure to the treat in which naïve B cells are involved; and the secondary immune response that involves other exposures to the same treat, involving memory B cells.[1]

The T lymphocytes are mediators of cell-mediated immunity. When the pathogen is engulfed by phagocytes, the antibodies cannot recognise it or other intracellular microbes. For this, there are two subtypes of T Lymphocytes.

$CD4^+$ T Cells, also known as helper T lymphocytes are known to support the activity of other immune cells. They can activate macrophages and enhance their microbicidal activity; help B cells produce antibodies; and even recruit neutrophils, eosinophils and basophils to the site of infection and/or inflammation through cytokines and chemokines production.[12] This function occurs in parallel with other two T cell activation signals, specifically the antigen-presentation by the MHC Class II present at the surface of antigen-presenting cells and costimulation.[1,6] There are 4 distinct subsets of $CD4^+$ T cells: 1) Th1 cells can produce cytokines, being Interferon-gamma (IFNγ) one of the most important. They lead to the activation of macrophages and increase their microbicidal activity; they also have an important role in the resistance to bacterial infections and are responsible for the induction of several autoimmune diseases, such as inflammatory bowel disease (IBD);[12] 2) Th2 cells mediate the host defence against extracellular parasites and are responsible for the induction of allergic inflammatory diseases and asthma;[12] 3) Th17 cells are responsible for mediating the immune response against extracellular bacteria and fungi, has also participation in autoimmunity diseases;[12] and finally 4) Treg Cells, which are responsible for self-tolerance, lymphocyte homeostasis and regulation of immune responses.[12]

$CD8^+$ T Cells (or cytotoxic T lymphocytes) recognize and bind via its T cell receptor (TCR) to MHC Class I, a glycoprotein present in all nucleated cells, making these cells able to identify intracellular antigens, infected or altered cells (e.g. tumour cells), and consequently, killing and eliminating the reservoir of infection (Figure 1.2).[1,6,7,13]

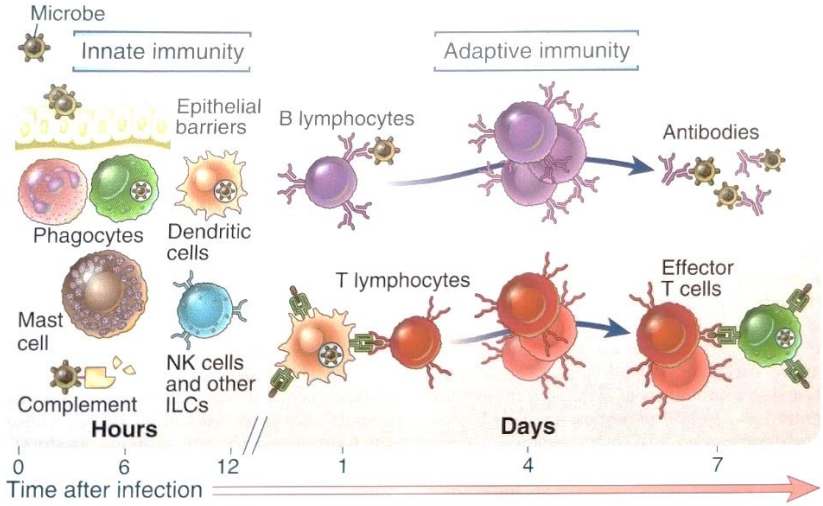


Figure 1.2 - Innate and adaptive immunity: *the mechanisms of innate immunity provide the initial defence against infections. Adaptive immune response develop later and require the activation of lymphocytes. The kinetics of the innate and adaptive immune responses*[1]

1.1.4 Fibroblasts and their interaction with the immune system

Fibroblasts are the most common cell type of connective tissue. The main role of fibroblasts in wound healing and these cells can define the architecture of tissue through depositing and remodelling extracellular matrix components (ECM).[15,16] To control the process of tissue architecture and percentage of the matrix, they induce matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs).[17] When the scaring process leads to excessive accumulation, proliferation and activation of fibroblasts and myofibroblasts, combined with excessive deposition of collagen and ECM, it is denominated as fibrosis.[16] Fibrosis is triggered by inflammatory and immunological reactions, where both the innate and adaptive immune system are involved (Figure 1.2). Extensive tissue damage, infection, autoimmune disease, or tumours can lead to a chronic inflammation process, which leads to fibrotic disease.[16]

There are several types of fibroblasts, all of them somehow participate directly or indirectly in the inflammatory process and immune response.[15]

The focus of this thesis is to understand the interaction between dermal fibroblasts and the immune system. The dermal fibroblasts are essential components of the skin, due to its importance they are potentially used in cell therapies.[18,19] These cells have several described interactions with cells of the immune system, such as, dendritic cells, mast cells, macrophages, and keratinocytes, that have an important role in regulating skin physiology. They also have an important role in tissue homeostasis, inflammatory response regulation and engagement of leukocytes.[19] For example, Yin Z. described the role of fibroblasts in autoimmune diseases. Dermal fibroblasts can secrete insulin-like growth factor-I and certain cytokines which contribute to hyperproliferation of psoriatic skin.[20] Due to the direct interaction of fibroblasts with immune cells, supporting the immune response and inducing inflammation, these cells are considered as part of the immune system.[19] Figure 1.2 exhibits the complex interaction previously described and the direct and indirect connection with the different blood cells and the cytokines, growth factors and enzymes underlying these interactions, demonstrating their role in the immune system.

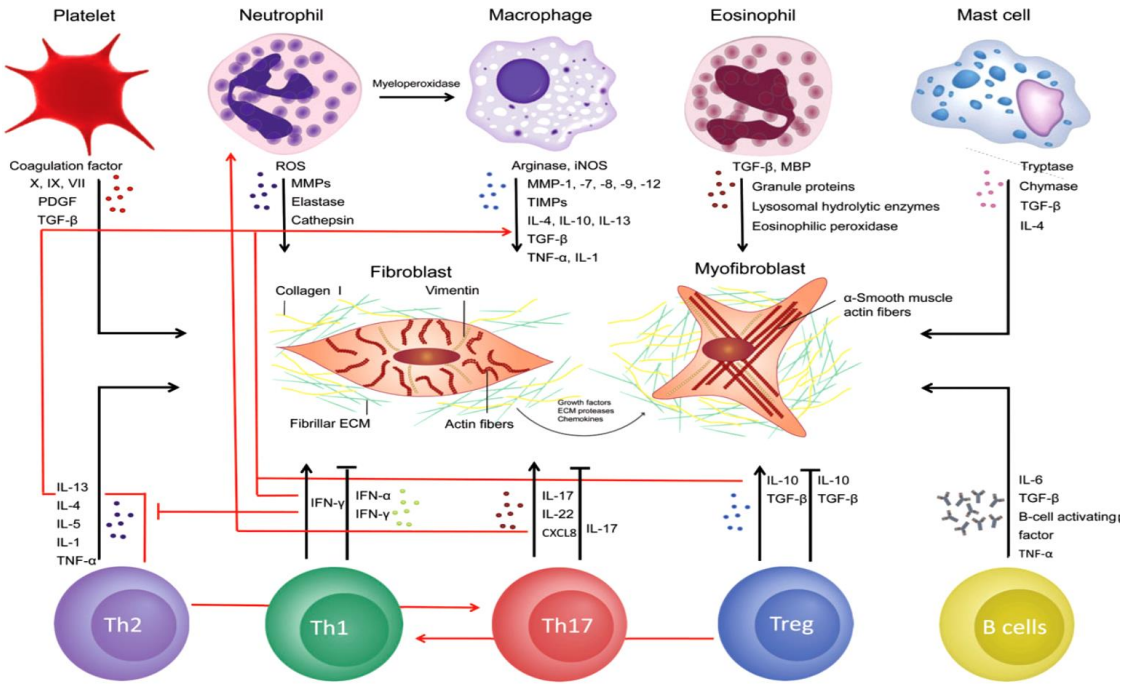


Figure 1.3 - Impact of components of the innate and adaptive immunity on the activation of fibroblasts. *Cytokines, growth factors, and enzymes released by immune cells directly (black arrows) promote fibroblast activation and indirectly (red arrows) lead to myofibroblast activation via further induction of pro-inflammatory, pro-fibrotic factors in other immune cells. IFN: interferon; IL: interleukin; iNOS: inducible nitric oxide synthase; MBP: major basic protein; MMP: matrix metalloproteinase; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; TGF: transforming growth factor; TIMP: tissue inhibitor of matrix metalloproteinase; TNF: tumour necrosis factor.* [16]

1.2 Chronic inflammation

Inflammation is a defence mechanism, triggered by the innate immune system, in response to tissue or cell damage, and can be induced by chemical, physical or microbial agents.[3] This mechanism recruits leukocytes to the site, increasing permeability of blood vessels resulting in local redness, swelling and elevated temperature.[21,22]

The process of inflammation has different stages, the inflammatory response that aims wound healing is known as acute inflammation[23,24] being essential for the prevention of infectious diseases.[3] In this stage, the cells of the immune system migrate to the injury site and become activated with increased expression of activation molecules and cytokines.[23] When this process persists, it leads to chronic inflammation, which can cause severe tissue deterioration and cause several diseases.[25] This is a process underlying a significant percentage of disease-related deaths worldwide, with more prevalence in elder patients. [25,26] Aging is associated with a decrease in the correct function of innate and adaptative immune system[27,28] and chronic inflammation is part of several pathologic processes in several age-related disorders, namely atherosclerosis, sarcopenia, osteoporosis, diabetes, cardiovascular diseases, cancer, among others.[21,26,29]

1.3 Cell Activation

1.3.1 Mitogens

A mitogen is a peptide or small protein that induces a cell to begin cell division: mitosis. During the normal process of cell function, human T cells are activated through phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2) hydrolysation, that induce the production of inositol triphosphate (IP_3) responsible for releasing intracellular calcium (Ca^{2+}). The degradation of PI(4,5)P2 and the diacylglycerol (DAG) that activates lymphocytes and the protein kinase C (PKC) activity.[30]

Phorbol 12-myristate-13-acetate (PMA), derivative from phorbol ester, mimics the DAG, this molecule is an activator of (PKC) activity.[31–33] In immune function assays, PMA is widely used as a mitogen in combination with Ionomycin, an ionophore of Ca^{2+} that works in synergy in enhancing the activation of PKC.[33,34]

1.3.2 Cytokines and other signalling molecules

Cytokines are soluble signalling proteins produced by different types of immune cells, that mediate and regulate immunity, inflammation, and haematopoiesis. These molecules are related to deregulatory processes, such as acute infections, autoimmune reactions, hypersensitivity, tissue injury, among others.[35] There are pro and anti-inflammatory cytokines that are a dynamic part of the inflammatory response, being released at the site of inflammation, these molecules facilitate the intervention of cells of the immune system in the healing process.[26]

In this thesis, we assessed two different cytokines, both with a role in inflammation and immune response, and also a signalling molecule.

The interferon-gamma (IFNγ), a glycosylated protein produced by NK cells, $CD4^+$ and $CD8^+$ T cells, with immunoregulatory properties, namely macrophages activation and increased expression of immunoglobulins on phagocytes. It also has an important role in immunological cell signalling and a main component in regulatory protein, being crucial to inflammatory response and cell-mediated immune responses.[36] IFNγ is composed by two chains, one responsible for binding and another responsible for signalling and receptor machinery for signal transduction. [35,37] With binding properties, such as binding motifs for the Janus tyrosine kinase (Jak)1 and the latent cytosolic factor, signal transducer and activator of transcription (Stat)1[38], this connection is essential for receptor phosphorylation, signal transduction, and induction of biological response.[38]

The interleukin 6 (IL-6), is a glycoprotein produced by different types of immune cells and even by fibroblast[39], involved in the inflammatory process, like injuries,[28] and in the early response to infection.[40] It also can stimulate the proliferation of B cells, regulate the concentration of acute-phase proteins after infection, and intervene in the production of immunoglobulin A.[25,35,41] Although the extent of the connection between fibroblasts and the immune system is briefly known, it has been described that the fibroblasts are able to produce il-6 during inflammatory processes[39], and for that reason, it was decided to include this cytokine in this thesis.

The Fibroblast growth factor-2 (FGF2) is a pleiotropic heparin-binding factor, acting in different types of cells, such as endothelial cells. It is a signalling molecule able to promote growth and differentiation in a wide-ranging of cell types[42]. FGF2 is connected with the angiogenesis process and can induce a complex 'pro-angiogenic phenotype' in endothelial cells responsible for neovascularization.[43,44] It has been demonstrated that elevated levels of FGF2 are directly connected with deregulated angiogenic and inflammatory response related to several diseases, including cancer.[42] Due to this connection, it was decided to include this signalling molecule in this thesis.

1.3.3 Cell surface biomarkers

Cell surface marker expression has been described for the identification and isolation of many cell types.[45] In this thesis different cell surface markers were used, whose function is described in Table 1.1.

Table 1.1 - Cell surface markers utilised in this thesis assays and their especifications[1,46–49]

Cell Surface Marker	**Molecular Structure**	**Main Cellular Expression and functions**	**Application in this thesis**
CD3	Ig Superfamily	Expressed in the cell surface of T-Lymphocytes and signal transduction of T-cell antigen receptor	T-lymphocytes marker
CD4	Ig Superfamily	Class II MHC-restricted T-Lymphocytes. Also, expressed in monocytes, macrophages, and dendritic cells.	T-lymphocytes marker
CD69	C-Type II lectin	An early marker of lymphocytes activation and expressed in lymphoid tissue.	T-lymphocytes activation marker
CD154 (CD40L)	TNFR superfamily	Ligand for CD40. Activation of B cells, macrophages, and endothelial cells. Expressed in activated CD4+ T cells involved in the pro-inflammatory response.	Cell surface marker for inflammatory response

1.4 Aims of the thesis

Inflammatory chronic diseases are characterised by relatively slow development and non-specific symptoms, making them challenging to diagnose, and leading to delayed treatment and an inevitable disease progression. It is imperative to identify and manage the underlying conditions to slow disease progression and reduce the likelihood that costly comorbidities will develop.[50]

In inflammatory diseases that lead to tissue damage and loss of function, the actual diagnosis is lengthy and performed through several patient samples. The elucidation of whether an inflammatory mechanism is taking place is critical, but ideally, it should be identified through minimally invasive procedures, such as analysis of blood samples or skin biopsies (fibroblasts). To achieve this, it is important to have a relevant panel of inflammatory biomarkers that provides a straightforward indication of chronic inflammation.

The current work aims to optimise a protocol to search for biomarkers of chronic inflammation from blood cell and fibroblast. We hypothesised that this could be addressed by direct stimulation of samples with a mitogen or cytokines. Different concentrations of mitogens (PMA with Ionomycin) were tested, as well as pro-inflammatory cytokines and LPS to obtain effective cellular stimulation in the shortest time possible, preserving cell viability. The stimulation was followed by the evaluation of the expression of cell surface activation markers, CD69 and CD154 by flow cytometry. For the evaluation of the pro-inflammatory cytokine (IL-6, IFNγ), flow cytometry and RT-PCR were performed.

The project was included in a broader project lead by our group that aims to understand how inflammation is implicated in Congenital Disorders of Glycosylation (CDG), a rare, chronic multi-organ disease, where inflammation may have a role.

2. Materials and methods

2.1 Stimulation

2.1.1 Whole blood stimulation

The present protocol aims at testing the activation of peripheral blood T Cells through stimulation of blood samples. The samples consisted of (1) buffy coats of healthy male volunteers between 20 and 40 years old, provided and ethically approved by the Portuguese Blood and Transplantation Institute and (2) whole blood collected in Monovette Li-heparin tubes (Sarstedt) from a volunteer with 54 years old healthy female obtained with consent for experimental purposes. The samples were diluted in RPMI medium (Gibco) enriched with 10% of fetal bovine serum (FBS, Gibco), 1% of L-glutamine and 1% of penicillin/streptomycin, in a 1:4 proportion. For the stimulation, PMA and Ionomycin were added in different concentrations (Table 2.1). In the samples destined for intracellular cytokine staining, 0,2% Brefeldin-A was added before stimulation to block protein transport. After stimulation, the cells were incubated for 5 hours at 37 ºC under slight agitation (85 rpm) to avoid cell precipitation. After the incubation time, Red Blood Cells Lysis Buffer (RBCL) (BD BioLegend) was used according to the manufacturer instructions to remove blood cells from the samples. Then, cells were washed and resuspended with 1X Phosphate-buffered saline (PBS).

Table 2.1. - Concentration of PMA and Ionomycin for blood stimulation: *PMA and Ionomycin were the stimuli selected for this experiment. Each combination of the different concentrations was tested to define the best stimulus for future studies.*

Stimulus	PMA concentration	Ionomycin concentration
1	5,5 ng/ml	0,01 µg/ml
2	5,5 ng/ml	0,05 µg/ml
3	5,5 ng/ml	0,1 µg/ml
4	50 ng/ml	0,01 µg/ml
5	50 ng/ml	0,05 µg/ml
6	50 ng/ml	0,1 µg/ml

2.1.2 Stimulation of fibroblasts

Fibroblast cultures from a PMM2-CDG patient donor with consent for experimental purposes were previously obtained from skin biopsies from Professor Gert Martthijs from KU Leuven, Belgium. The cells were cultured at 37°C in a 5% CO2 humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco) containing 10% FBS, Gibco, 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco) in 6-well plates. After the cells

reached 80-90% confluence, three different stimuli were added, namely LPS, TNFα and PMA in combination with Ionomycin. The cells were incubated for 6h, in the same culture conditions. Cells were harvested and washed twice with PBS and the pellet was frozen at -20 ºC to perform RNA extraction and RT-PCR.

2.2 Flow Cytometry

Flow cytometry is a technique that allows a multi-parameter analysis of single cells in a suspension (Table 2.2).[51] It can measure different fluorescent parameters at the same time on the same cell.

Table 2.2 - Applications of flow cytometer analysis[51]

Applications of the flow cytometer
DNA/Cell Cycle analysis
Cell viability and proliferation
Intracellular ionic fluxes
Multicolour phenotyping (cell surface or intracellular)
Monocyte oxidative burst and monocytes phagocytosis
Neutrophil oxidative burst or Neutrophil phagocytosis
Microbiological analysis
Cell trafficking
Cellular and antibody or complement-mediated cytotoxicity
Sorting based on morphology (FSC or SSC) and/or fluorescent characteristics

This method is based on the principle of fluorescence, thus allowing to detect, evaluate and compare lymphocytes with fluorescent markers. The channel where the sample runs is denominated fluidic system and the component of the optical system of the flow cytometer, is where the laser light beam illuminates a single cell, this light excites the fluorochrome attached to the cell, which produces a fluorescence emission.[52] The reading of the signal sent is obtained in two different ways, through forward angle scatter (FSC) that provide the size of the cell and Side angle scatter (SSC) that analyses the internal structure, granularity, and surface texture. The photodetector, convert the signal into digital data and transferred to the software (Figure 1.3).[52,5354]

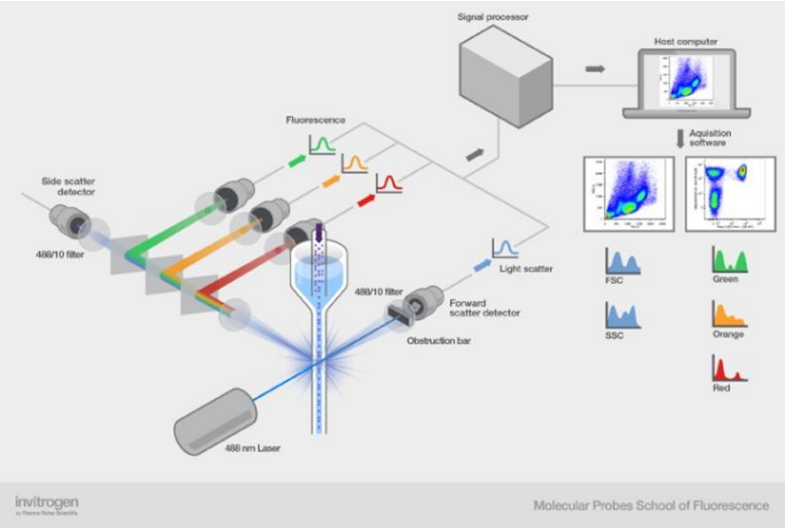


Figure 2.1 - The working parts of the flow cytometer: *This is the place within the flow cytometer where the laser light hits the individual particles as they pass in front of the laser, one at a time.*[53]

This technique was employed in this work to evaluate T cell activation, using the Attune® Acoustic Focusing Cytometer (Applied Biosystems). To analyse the obtained data was performed with FlowJo® software.

2.2.1 T cell activation analysis

Blood samples subjected to Red Blood Cell lysis were cell-surface stained with CD3, CD4, CD8, CD69 and CD40L (Table 2.3). To evaluate intracellular cytokines like IFNγ, cell fixation and permeabilization were performed with the BD cytofix/cytoperm kit (BD Bioscience). This step is important to make sure that we are able to evaluate the cytokines intracellularly. All the samples were resuspended with 300µl of 2% paraformaldehyde (PFA) to preserve viability and stored at 4ºC until data acquisition though using the Attune® Acoustic Focusing Cytometer (Applied Biosystems).

Table 2.3 - Panel of antibodies used in flow cytometry assays

The panel of antibodies used in flow cytometry assays	
Markers and cytokines	Fluorochromes
CD3	Perpcy 5.5
CD4	FITC
CD4	APC H7
CD69	FITC
CD69	Pe cy 7
CD154/40L	APC
IFNγ	FITC

2.3 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) - Evaluation of cytokine gene expression

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) is a powerful technology with the ability to amplify and detect mRNA. In this study, this technique was used to evaluate the expression of the cytokine IL-6 and the Fibroblast Growth Factor-2 (FGF2) in response to stimuli. This technique is extremely sensitive, and only needs a small amount of cDNA sample to amplify. It also has a high specificity and good reproducibility. [55,56] This technique resorts to a mix of target DNA with Taq Polymerase, primers, and nucleotides. PCR reaction starts when the mix is heated until 95°C, this helps to break the hydrogen bonds of DNA and lead to two denatured chains. The temperature is reduced to 60°C to induce the chains to rejoint allowing the primers (small DNA sequences that define the start and ending position of the segment of DNA to amplify also known as probes), to bind the complementary position of DNA chain. Then the temperature is raised again to 95°C so Taq polymerase starts synthesizing the DNA creating a copy of the sequence of interest. [57] This cycle is repeated 40 to 50 times, and therefore thousands of copies are obtained. In RT-qPCR, amplified copies are immediately measured as the reaction occurs. The obtention of the cycle threshold (Ct) - cycle number when the fluorescence of a PCR product can be detected above the background signal - allows quantifying the relative expression for each gene submitted for evaluation.

For the analysis of the genetic expression of cytokine IL-6 and FGF2 after stimulation of fibroblasts, ribonucleic acid (RNA) extraction was performed following its conversion to cDNA. The RNA extraction was performed resorting to GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich)[58] (appendix I). The cell lysis solution consisted of a 1:100 mixture of β-mercaptoethanol (Sigma) and the lysis solution provided by the kit. 250 µl of this mixture were added throughout pipetting to each sample and cells were lysed. The lysate was filtered resorting to a filtration column by centrifugation at 12000g for 2 minutes at 4ºC (all the next centrifugation steps were performed in the same conditions). 250 µl of 70% ethanol were added to the filtered lysate, thoroughly mixed, and added to a binding column whereas the RNA stayed attached after centrifugation. This column was washed with 250 µl of washing solution 1 by centrifugation. Followed by two washes with 500 µl of washing solution 2 by centrifugation. The remaining ethanol was removed by centrifugation of the column without any volume. The column was placed into a clean collection tube and RNA was eluted with 55 µl of elution solution by centrifugation. The flow-through was collected and added again to the column to elute the remaining RNA by centrifugation. Since RNA is not chemically stable, the conversion of purified RNA to cDNA was immediately performed. The next step was carried out using the High-Capacity

cDNA Transcription Kit (Applied Biosystems). Per each sample, 50µl of reaction mix for the conversion was added. This mix is composed by 10µl of RT buffer, 10µl of random primers 10X, 4µl of 100mM deoxynucleoside triphosphates (dNTPs), 7,5 µl of reverse transcriptase and 18,5 µl of RNAse free water (NZYTech). For each sample, 50 µl of purified RNA and 50 µl of the mix was added in a PCR tube. The conversion of RNA was achieved using the program described in Table 2.4, on a thermocycler (MJ Research), and the cDNA obtained was stored at -20ºC.

Table 2.4 - cDNA synthesis PCR program on a thermocycler

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (ºC)	25	37	85	4
Time	10 min	120 min	5 sec	∞

The RT-qPCR reaction was carried out in 0,1 ml PCR tubes (Simport) in a total volume of 10 µl. The reactions were prepared in duplicate by adding 2 µl of cDNA, 5 µl of TaqMan Fast Universal PCR Master Mix 2x, 2 µl of the diluted probe and 1 µl of RNAse free H_2O (Applied Biosystems). The probes included β-actin and GAPDH as the endogenous controls, IL-6 and FGF2, the target genes. For this experiment, the Rotor-Gene 6000 Series (Corbett) was used with the conditions described in Table 2.5.

Table 2.5 - RT-qPCR reaction conditions

	Cycle 1	**40-50 cycles**	
Temperature (°C)	95	95	60
Time (seconds)	20	3	30

Gene expression was assessed by the Cycle Threshold (CT) method.[59] This method provides, through CT values, the relative expression and relative quantification values of the gene in interest. The values to determine relative expression can be obtained through the mean of gene expression of the target gene against the mean expression of the endogenous genes and resorting to the adapted equation $2^{\Delta CT}$*1000. The relative quantification can be obtained through ΔΔCT, that is the mean of a ΔCT of treated sample against the ΔCT of untreated sample and resorting to the $2^{-\Delta\Delta CT}$*1000 equation. The CT values were determined using the Rotor-Gene 6000 series software (version 1.7) and further calculations were performed using Microsoft Office Excel®.

3. Results and discussion

3.1 Blood stimulation analysis

3.1.1 Optimization of stimulation

To optimize the protocol of whole blood stimulation and T cell activation analysis we resorted to flow cytometry and FlowJo© software. Data was analysed resorting to the following gating strategy: first, through the evaluation of the FSC and SSC where it is possible to identify the lymphocytes and to apply a gate to the cells (Figure 3.1); secondly, single cells were selected taking into consideration the linearity of the height and area of single cells that pass through the cytometer flow, and thereby removing any non-single cells (doublets or clumps) (Figure 3.1.). This gating strategy was used in cell activation analysis.

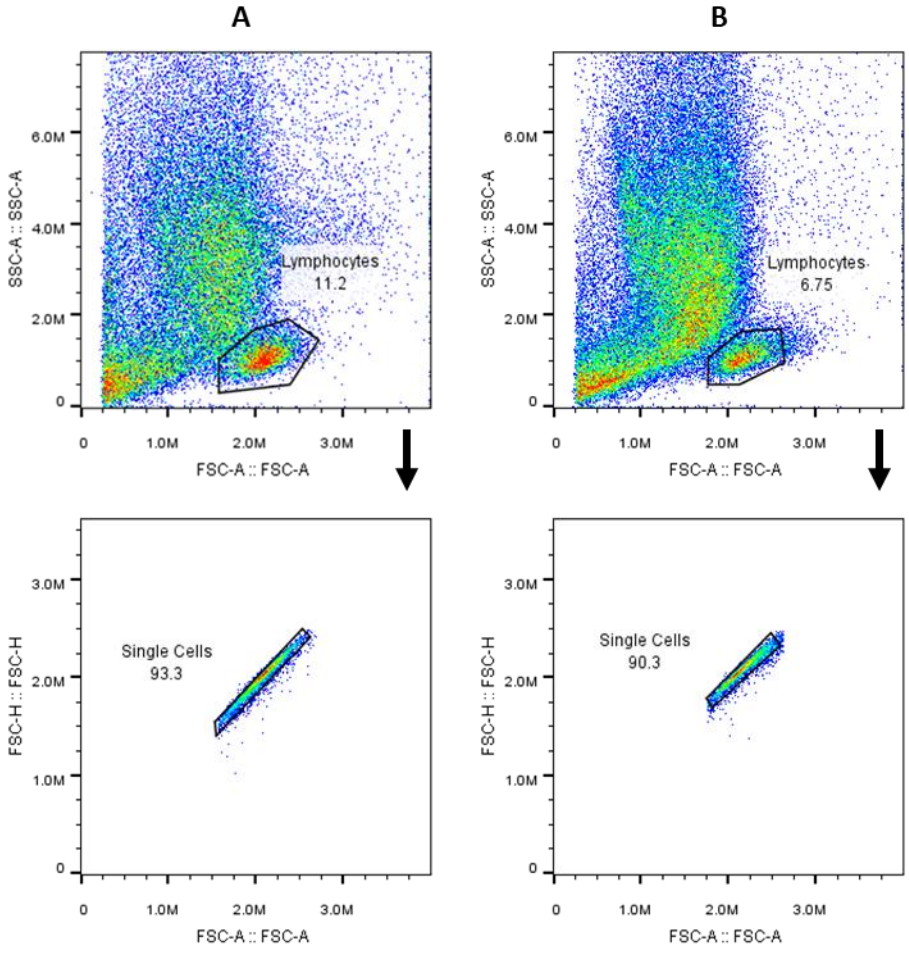


Figure 3.1 - Gate strategy *- Gating strategy of the unstimulated sample (A) and the stimulated sample (B). In the first graph is represented the gate on the lymphocytes based on the FSC/SSC; In the second graph, taking into consideration the linearity of the height and area, single cells are selected.*

After single cells selection, CD69 was analysed through the corresponding channel, BL2-A (Figure 3.2). The graphics show the fluorescence intensity of the signal from CD69-positive cells compared with the unstained cells. This strategy was applied to every sample.

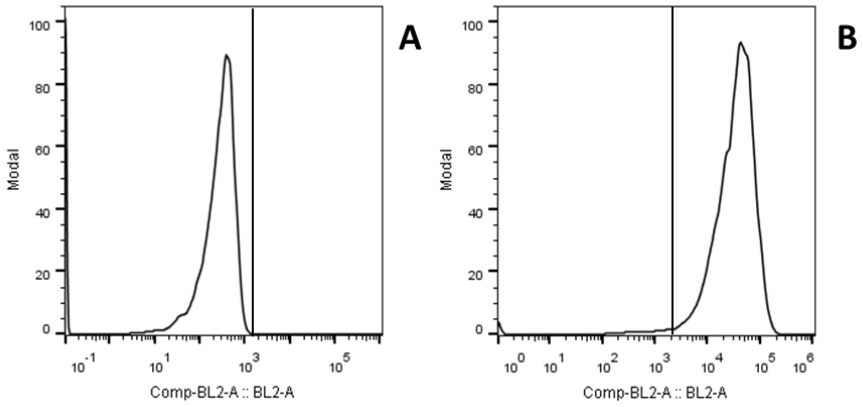
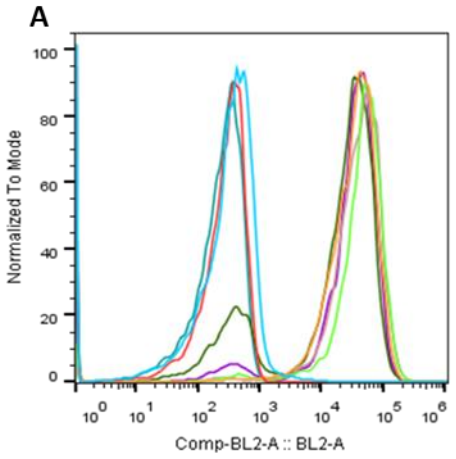


Figure 3.2 - Fluorescence intensity signal obtained for the cell surface marker CD69*: Histogram represents the intensity of the signal, as evaluated by flow cytometry, from the unstained cells (A) with a negative response compared with the stimulated sample with CD69-positive cells (B).*

To perform the optimization of stimulation and determine the most adequate stimulus to obtain a good signal keeping cell viability, 6 different concentrations of the stimulus were tested (Figure 3.3)



B

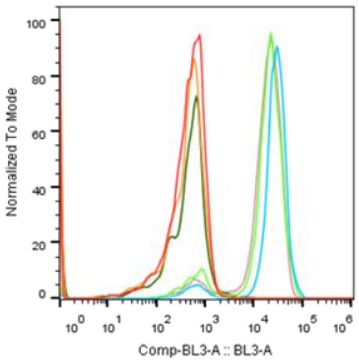
	Sample Name	Subset Name	Count	Median : Comp-BL2-A
	Specimen NE.fcs	Single Cells	7012	273
	Specimen US.fcs	Single Cells	5105	261
	Specimen E1.fcs	Single Cells	4296	35063
	Specimen E2.fcs	Single Cells	4485	46122
	Specimen E3.fcs	Single Cells	1612	40146
	Specimen E4.fcs	Single Cells	5541	22582
	Specimen E5.fcs	Single Cells	6739	208
	Specimen E6.fcs	Single Cells	5823	33896

Figure 3.3 – CD69 expression in different blood samples before and after stimulation: A - *Fluorescence intensity signal obtained after staining with anti-CD69 and analysis by flow cytometry. Comparison between the unstimulated and unstained samples. It was observed a positive response in the different stimuli except stimulus 5. The scheme colour can be consulted in the following table (B).* ***B -****The description at the right details the cell count and median fluorescence intensity (MFI) from each sample. The colour of each histogram corresponds to the colour used to identify each specimen. E1: 5,5 ng/ml PMA and 0,01 µg/ml Ionomycin; E2: 5,5 ng/ml PMA and 0,05 µg/ml Ionomycin; E3: 5,5 ng/ml PMA and 0,1 µg/ml Ionomycin; E4: 50 ng/ml PMA and 0,01 µg/ml Ionomycin; E5: 50 ng/ml PMA and 0,05 µg/ml Ionomycin; E6: 50 ng/ml PMA and 0,1 µg/ml Ionomycin.*

In figure 3.3, we can observe the cell count, which shows the number of cells acquired in each sample and the median fluorescence intensity (MFI). The ideal stimulus choice was based on these two parameters. Unlike expected and considering the two parameters the stimulus 5 was not performed correctly. So, this data was removed for evaluation of the results and conclusions. It was observed that the ideal stimulus for evaluation of whole blood samples is stimulus 2 with 5.5 ng/ml of PMA and 0.05 µg/ml of Ionomycin for detection of cell surface markers.

In a second optimization, the protocol was adapted to include stimulation of cytokines and cell surface markers together. The following graph indicates the results for the CD3 marker (Figure 3.4). This is a marker for T lymphocytes, which indicates that we are analysing only the cells of interest. As we can see we have a high fluorescence intensity and median in all marked samples, whether they are stimulated or not, showing the presence of lymphocytes. We can also observe the fluctuation in number of cells, this can be due to minor number of cells that were marked.

A



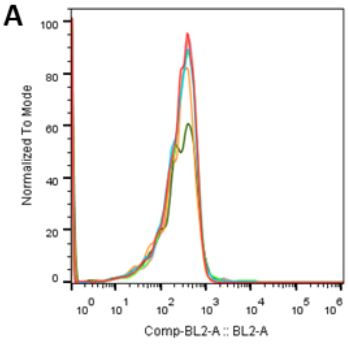
B

	Sample Name	Subset Name	Median : Comp-BL3-A	Count
	Nest US.fcs	Single Cells	454	5070
	Nest CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	26386	3904
	Estimulo1 US.fcs	Single Cells	423	806
	Estimulo1 CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	19392	2790
	Estimulo2 US.fcs	Single Cells	448	291
	Estimulo2 CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	18810	4152

Figure 3.4 – CD3 expression in different blood samples: ***A -*** *Fluorescence intensity signal obtained with CD3+ cells; Comparison between the unstimulated and unstained samples with all stimulus. It was observed a positive response in a different stimulus.* ***B -*** *The description at the right details the median fluorescence intensity from each sample and cell count. The colour of each histogram corresponds to the colour used to identify the specimen. E1 - 5,5 ng/ml PMA and 0,05 µg/ml Ionomycin; E2 - 50 ng/ml PMA and 1 µg/ml Ionomycin*

The analysis performed to the CD69 marker was not as successful as expected (Figure 3.5), this event can be due to errors in stimulation, such as the insufficient volume of stimulus used or even the lack of use of stimuli. And also is explained because brefeldin A was added before stimulation. According to *S. Nylander, I. Kalies*r brefeldin blocks protein secretion at an earlier step preventing the transport from the endoplasmic reticulum (ER) to the Golgi, and being an early activation marker, CD69 is trapped within the cell resulting in loss of surface expression.[60]

A



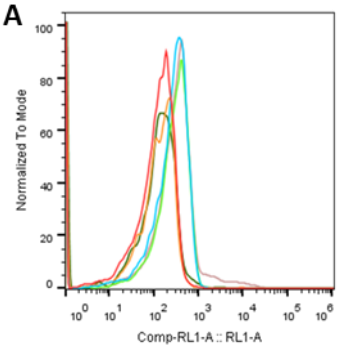
B

	Sample Name	Subset Name	Count	Median : Comp-BL2-A
	Nest US.fcs	Single Cells	5070	255
	Nest CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	3904	247
	Estimulo1 US.fcs	Single Cells	806	245
	Estimulo1 CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	2790	269
	Estimulo2 US.fcs	Single Cells	291	222
	Estimulo2 CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	4152	276

Figure 3.5 - Fluorescence intensity signal obtained: A- *Comparison between the unstimulated, unstained and CD69+ cells samples with two different stimuli. It was observed to have no response in all samples.* ***B*** *- The description at the right details the median fluorescence intensity from each sample and cell count. The colour of each histogram corresponds to the colour used to identify the specimen. . E1 - 5,5 ng/ml PMA and 0,05 µg/ml Ionomycin; E2 - 50 ng/ml PMA and 1 µg/ml Ionomycin*

In the evaluation of cytokines, we can observe that although the fluorescence intensity emitted from IFNγ is not high, the most appropriate is stimulus 2 (50 ng/ml PMA and 1 µg/ml Ionomycin), contrary to the stimulus used for stimulation of surface markers.

A



B

	Sample Name	Subset Name	Median : Comp-RL1-A	Count
	Nest US.fcs	Single Cells	96.1	5070
	Nest CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	245	3904
	Estimulo1 US.fcs	Single Cells	107	806
	Estimulo1 CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	264	2790
	Estimulo2 US.fcs	Single Cells	105	291
	Estimulo2 CD69PECD3percpcy55IFNyAPC.fcs	Single Cells	284	4152

Figure 3.6 *-* ***IFNγ expression in cells: A -*** *Comparison between the unstimulated and unstained samples with all stimulus. It is observed a positive response in a different stimulus;* ***B*** *- The description at the right details the median fluorescence intensity from each sample and cell count. The colour of each histogram corresponds to the colour used to identify the specimen. E1 - 5,5 ng/ml PMA and 0,05 µg/ml Ionomycin; E2 - 50 ng/ml PMA and 1 µg/ml Ionomycin*

3.1.2 Protocol optimization with the evaluation of new markers

Once the appropriate stimulus was found, a new analysis was performed with a different surface cell marker, CD154, also known as CD40L, a marker predominantly expressed on activated CD4+ T cells involved in a pro-inflammatory response.[47,48] In order to obtain a more precise and selective analysis, a new gate strategy was applied in this next analysis. With this gate, we obtain only T lymphocytes, the cells under study. Single cells were first selected (Figure 3.7A) to exclude doublets and clumps. And next, a quadrant gate was applied based on CD3 (RL2-A) versus CD4 (BL3-A). This gate allowed selecting the cells of interest CD3 + CD4 + cells (i.e., T lymphocytes (Figure 3.7. B - Q2)). Once selected quadrant 2 (Q2 – T lymphocytes), a

histogram was obtained with the fluorescence intensity for the channel of interest, in this case, CD69 (BL4-A) (Figure 3.7 C). This gating strategy was used to perform the next cell activation analysis.

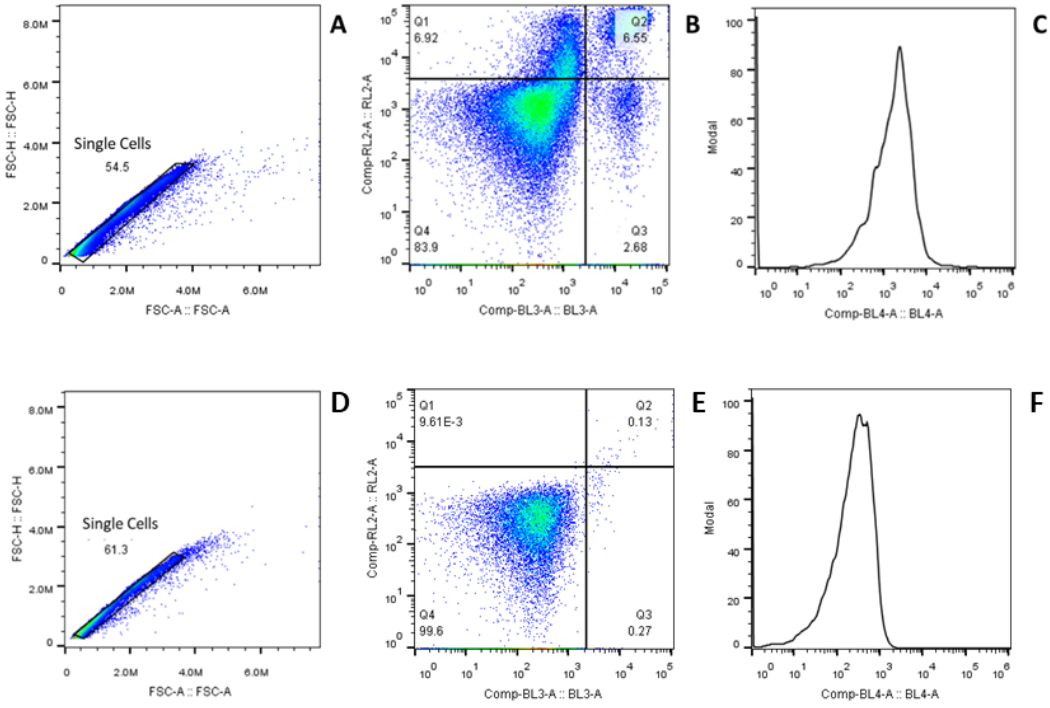
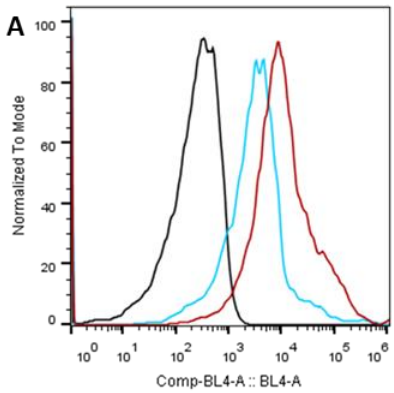


Figure 3.7 - Gating strategy applied in the stimulated sample (A,B,C) vs unstained (D,E,F) for the evaluation of new cell surface markers: A and D – *Evaluation of the FSC and SSC where it is possible to identify and apply a gate to the single cells;* ***B and E –*** *Analysis of the CD3 (RL2-A) with CD4 (BL3-A) where the quadrants were divided, and positive CD3 and CD4 quadrant (Q2) was selected;* ***C and F –*** *Histogram with the CD154 (BL4-A) fluorescence intensity obtained from Q2.*

After the selection of the gate/quadrant of interest, the analysis of the fluorescence intensity was performed. For each marker, three samples were compared, namely unstained (US), Non-stimulated (NS) and stimulated (E1) (Figure 3.8 A). To compare the different samples, the MFI was used, it is possible to observe that, as predicted, the intensity of the fluorescence emitted by the stimulated sample (E1) is higher than the non-stimulated and unstained samples (Figure 3.8 B) for the CD69 activation marker which means that T cells are being successfully activated following stimulation.

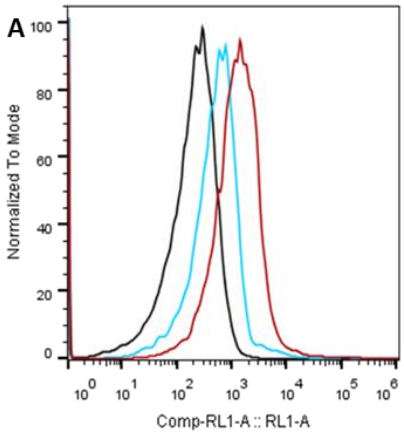


B

	Sample Name	Subset Name	Count	Median : Comp-BL4-A
☐	Specimen E1 5H.fcs	Q2: BL3-A+ , RL2-A+	11619	8377
☐	Specimen NE 1.fcs	Q2: BL3-A+ , RL2-A+	7177	1176
☐	Specimen US.fcs	Lymphocytes	31211	22,0

***Figure 3.8 - Fluorescence intensity signal obtained with CD69: A** - Comparison between the unstimulated, unstained and CD69+ cells samples **B** - The description at the right details the median fluorescence intensity from each sample and cell count. The colour of each histogram corresponds to the colour used to identify the specimen. E1 - 5,5 ng/ml PMA and 0,05 µg/ml Ionomycin.*

Employing the previous gate strategy (Figure 3.7), the fluorescence intensity was evaluated for the CD154 marker. As previously seen for CD69, the MFI was very high compared to the unstimulated sample (Figure 3.9).



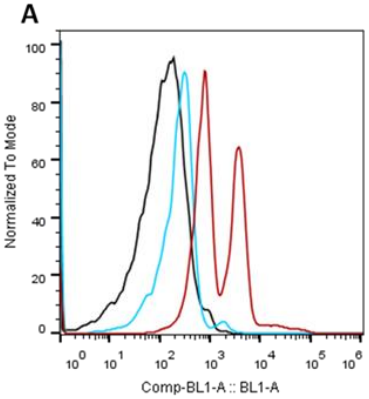
B

	Sample Name	Subset Name	Count	Median : Comp-RL1-A
☐	Specimen E1 5H.fcs	Q2: BL3-A+ , RL2-A+	11619	1141
☐	Specimen NE 1.fcs	Q2: BL3-A+ , RL2-A+	7177	467
☐	Specimen US.fcs	Lymphocytes	31211	158

***Figure 3.9 - Fluorescence intensity signal obtained with CD154: A** - Comparison between the unstimulated, unstained and CD154+ cells samples with two different stimuli. **B** - Cell count and MFI from every sample. E1 - 5,5 ng/ml PMA and 0,05 µg/ml Ionomycin.*

In this experiment, cytokines analysis following stimulation was performed separately so as not to block the expression of cell surface markers. The same gating strategy was applied (Figure 3.7)

A



B

	Sample Name	Subset Name	Count	Median : Comp-BL1-A
☐	Specimen E2 5H.fcs	Q2: BL3-A+ , RL2-A+	4308	963
☐	Specimen NE (E2).fcs	Q2: BL3-A+ , RL2-A+	1753	218
☐	Specimen US.fcs	Lymphocytes	31211	50,9

Figure 3.10 - Signal obtained with IFNγ + cells: A - *Comparison between the unstimulated and unstained samples with all stimulus. It is observed a positive response in a different stimulus. With better results in stimuli 2.* ***B –*** *Count and MFI from every sample. E2 - 50 ng/ml PMA and 1 µg/ml Ionomycin*

Contrary to the previous experiment, the IFNγ expression has a much higher fluorescent intensity (Figure. 3.10). We can see that there are several peaks, this can be explained by the fact that there are lymphocytes with higher intensity in the signal emission (Figure 3.11)

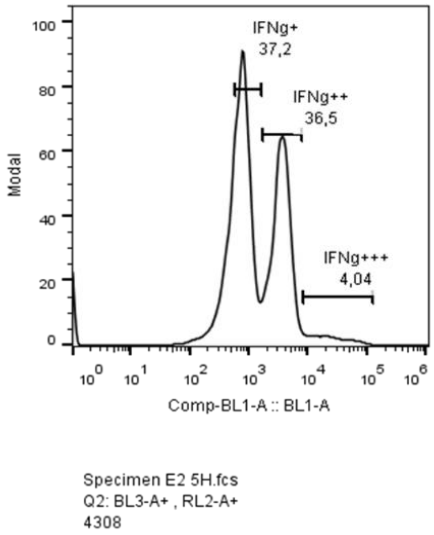


Figure 3.11 *- Histogram with the fluorescent intensity signal emitted by IFNγ. Comparison of the different peaks.*

With the present results, we were able to develop two different protocols. The first, for cytokine evaluation following blood stimulation, with a stimulus of 50 ng/ml of PMA and 0,1 µg/ml of ionomycin and the second, for evaluation of cell surface markers with a stimulus of 5,5 ng/ml of PMA and 0,05 µg/ml of ionomycin.

3.2 Fibroblasts stimulation

To optimize the protocol of dermal fibroblast stimulation analysis of data, we resorted to the analysis of gene expression, as evaluated by RT-qPCR. Data was analysed through CT values namely by the relative expression and relative quantification values of the genes of interest. Three different stimuli were used due to little support literature on fibroblast stimulation.[61] Different stimuli concentrations were tested to see if it can be used in the first place and second the concentration to be used in future studies (Table 3.1).

Table 3.1 – Stimuli and concentration tested

LPS	TNF	PMA with Ionomycin
0,0001 µg/ml	0,1 ng/ml	5 ng/ml – 6 µg/ml
0,001 µg/ml	1 ng/ml	25 ng/ml - 3 µg/ml
0,01 µg/ml	10 ng/ml	50 ng/ml - 1,5 µg/ml
0,1 µg/ml	100 ng/ml	100 ng/ml - 0,75 µg/ml
1 µg/ml	1000 ng/ml	200 ng/ml - 0,15 µg/ml
10 µg/ml	-	-

To evaluate the results, 1) the relative quantification was used that relies on the comparison between the expression of a target gene versus a reference gene or endogenous genes[62] and 2) the relative expression was also used that describes the change in expression of the target gene relative to some reference group such as an untreated control or a sample at time zero in a time-course study.[59] As endogenous control, β-actin and GAPDH were used, and the target genes were IL-6 and FGF2. Once again, the chosen stimulus must be the one with the lowest concentration that presents a satisfactory result. Through the following graphics (Figure 3.12) we can see that fibroblasts overexpress the two genes of interest following stimulation with any of the three stimuli used. TNFα is the stimulus with the highest expression, which leads us to believe that this is the ideal stimulus for future studies, followed by the PMA in combination with ionomycin, which also has a high expression of IL-6 and, finally, LPS, which has less expression compared to the other stimuli. It can also be determined that for the detection of IL-6 and FGF2 in dermal fibroblasts, a concentration of 100 ng/ml of TNFα, 25 ng/ml of PMA + 3 µg/ml of ionomycin and 1 µg/ml of LPS could be used for future experiments.

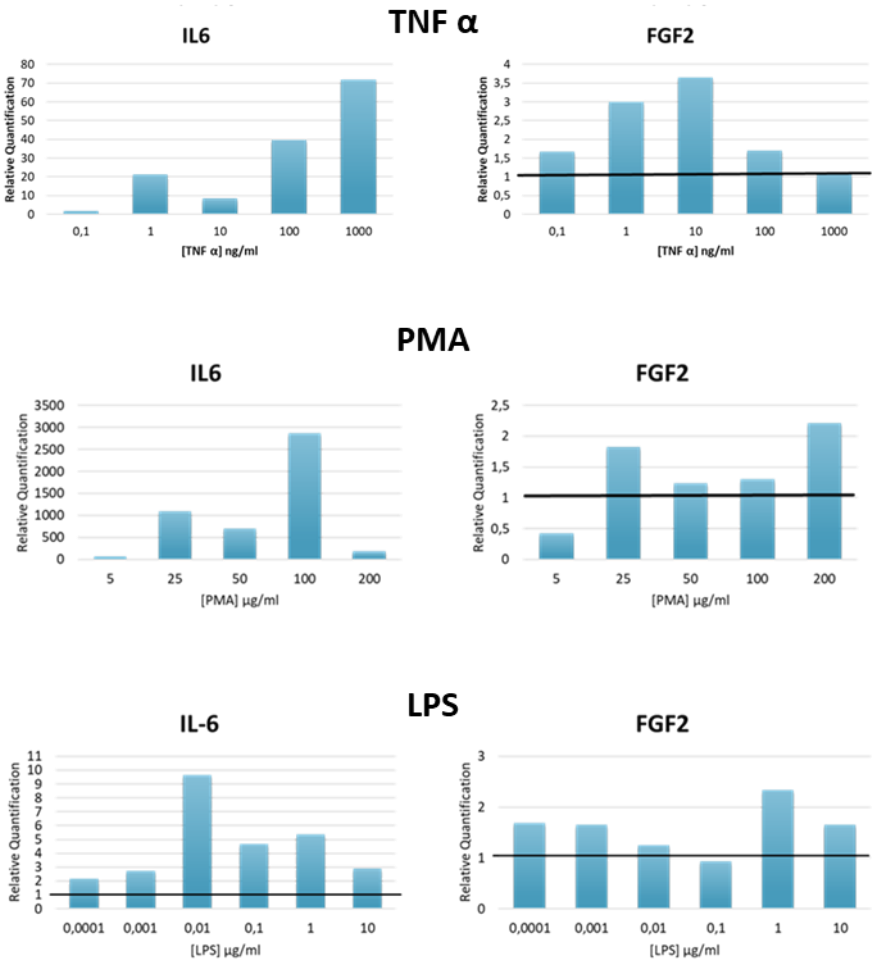


Figure 3.12 *-Relative quantification of IL-6 and FGF2 in fibroblasts. The cell was submitted to different concentration of stimuli TNF-α, PMA in combination with ionomycin and LPS. The expression of the IL-6 and FGF2 genes was evaluated by RT-PCR as referred to in the material and methods section*

4. Conclusion and future perspectives

Chronic inflammation can cause several diseases, exacerbate symptoms and contribute to a worse prognosis. Early identification of inflammatory processes is important to prevent this and also allow therapeutic interventions that may contribute to improving disease outcome. This work helps to demonstrate that a rapid protocol can be used to evaluate inflammatory biomarkers. In fact, when stimulated either leukocytes and fibroblasts were able to alter the expression of relevant cell surface biomarkers and cytokines. The analysis of such a response may allow the prediction of an inflammatory profile in patients, which can then be related to health outcomes such as physical functions and symptoms. These methods can be employed in samples obtained by minimally invasive methods such as blood samples and fibroblast. These protocols can be applied for comparing healthy patients versus unhealthy patients, to determine which cytokines and cell surface markers are differentially expressed and become a reliable biomarker of disease exacerbation or for prognosis.

In addition, the fact that dermal fibroblasts express pro-inflammatory cytokines, namely IL-6, reinforces that these cells have an active role in the inflammatory response in association with the immune system.[61] This interaction between dermal fibroblasts and the immune system can open a new field of research and can be an additional pathogenic mechanism of disease.

During the experiments, it was possible to improve the protocol through the results obtained, reaching an optimized final protocol.

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6. Appendix

6.1 Appendix I - GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich)

Experienced User Protocol

All spins at ; 14,000 × *g*.

Cultured Cells or Tissue

1 Release RNA from Cells or Tissues

- ❑ Add 2-mercaptoethanol to lysis solution (10 µL 2-ME/1 mL lysis solution).
- ❑ Lyse cells/homogenize tissue in 250 or 500 µL of lysis solution/2-ME mixture.
- ❑ Transfer lysate to filtration column. *Spin 2 minutes*. Discard filtration column.

2 Bind RNA to Column

- ❑ Add equal volume of 70% ethanol to filtrate (250 or 500 µL). Mix thoroughly.
- ❑ Transfer up to 700 µL lysate/ethanol mixture to binding column. *Spin 15 seconds*.
- ❑ Discard flow-through & repeat if necessary.

2 min.

15 sec.

3 Wash to Remove Contaminants

- ❑ Add 500 µL Wash Solution 1 to column. *Spin 15 seconds*.
- ❑ Transfer column to new collection tube.
- ❑ Add 500 µL Wash Solution 2 to column. **Note**: Ethanol must be added to Wash 2 concentrate before first use. *Spin 15 seconds*. Discard Wash Solution.
- ❑ Add second 500 µL Wash Solution 2 to column.
- ❑ *Spin 2 minutes* to remove ethanol.

15 sec.
15 sec.
2 min.

4 Elute Purified RNA

- ❑ Transfer column to new collection tube.
- ❑ Add 50 µL elution solution to column. *Spin 1 minute* (repeat if > 100 µg RNA expected).

1 min.

Pure Total RNA